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(71) Applicant: Yukijirushi Milk Industry KK

(72) Inventors: Hiroshi Tujibo (5-6-1, Onodai, Sayama-Shi, Osaka, Osaka-fu) and others

(74) Patent Attorney: Eiko Funabashi

(54) Title of the Invention:

PROTEASE-GENERATING BACTERIUM, A PROTEASE GENERATED BY THE BACTERIAL CELL AND A METHOD FOR PURIFYING THE SAME.

Claims:

1. A protease-generating bacterium of the genus Nocardiopsis[alkalophilic actinomycete; Nocardiopsis sp. strain OPC-210 (FERM P-10508)], where the cell wall is of meso type as determined by 2,6-diaminopimeric acid analysis and of a cell wall type III/type C and the phospholipid is of PIII type.

2. A purification method of a protease generated by a protease-generating bacterium, comprising recovering an acetone powder from the strain OPC-21 according to claim 1 and dissolving the acetone powder in a weakly acidic solution for dialysis and subsequent purification of the resulting dialyzate by column chromatography.

3. A method according to claim 2, where the purification by column chromatography comprises allowing the dialyzate to pass through a DEAE-Sephadex A-50 column equilibrated with the weakly acidic solution, for purification by CM-Sepharose CL-6B column chromatography.

4. A method according to claim 3, where the weakly acidic solution is an aqueous ammonium acetate solution.

5. A protease generated by the strain OPC-210 according to claim 1.

6. A protease recovered by a purification method according to claim 2.

Detailed Description of the Invention

Industrial Field of the Invention

The present invention relates to an alkalophilic actinomycete, more specifically a protease-generating bacterium, a protease generated by the bacterial cell and a purification method of the same.

Prior Art and Problems that the Invention is to Solve

Because bacteria of the genus Actinomycetes generate a wide variety of secondary metabolites, the bacteria are important bacteria. However, research works about the isolation and application of alkalophilic actinomycetes have generated only limited reports, when compared with research works about microorganisms growing in other abnormal environment.

For the purpose of the investigation and application of microorganisms growing in abnormal environment, in accordance with the invention, bacteria of alkalophilic actinomycetes have been isolated for the purification of an alkali protease generated by an alkalophilic actinomycete and the elucidation of the properties.

Means for Solving the Problem

The invention relates to a protease-generating bacterium sof the genus Nocardiopsis[alkalophilic actinomycete, Nocardiopsis sp. strain OPC-210 (FERM P-10508)], where the cell wall is of meso type as determined by 2,6-diaminopimeric acid analysis and of a cell wall type III/type C and the phospholipid is of PIII type, as well as a protease generated by the bacterial cell.

Further, the invention relates to a purification method of a protease generated by a protease-generating bacterium, comprising recovering an acetone powder from the strain OPC-21 and dissolving the acetone powder in a weakly acidic solution (preferably an aqueous ammonium solution) for dialysis and subsequent purification of the resulting dialyzate by column chromatography, where the purification by column chromatography preferably comprises allowing the dialyzate to pass through a DEAE-Sephadex A-50 column equilibrated

with the weakly acidic solution, for purification by CM-Sepharose CL-6B column chromatography.

The bacterium to be used in accordance with the invention was newly isolated from soil in Kyoto-Fu in November 1987. The bacteriological properties of the isolated strain (referred to as strain OPC-210 hereinafter) are now described below. Herein, the strain OPC-210 was deposited as FERM P-10508 at the National Institute of Bioscience and Human-Technology, the Agency of Industrial Science and Technology, MITI.

1. Morphological properties

The aerial hyphae are appropriately branched. Under general conditions, the aerial hyphae are not broken. The aerial hyphae grow very well in an agar culture medium with yeast and malt (ISP culture medium No. 2) and greatly promote spore formation. The branching of aerial hyphae is simple branching. No sporangia, sclerotium or flagellar spore are observed. At an early stage of spore formation, the aerial hyphae are observed in a more or less zigzag pattern; subsequently, the aerial hyphae are broken in long fragments and subsequently in spores in non-uniform sizes and with smooth surface. Under observation with scanning electron microscope, the spores are of a lemon shape, have a diameter of about 1 x 1.7 micron and a smooth surface.

Table 1

Culture medium	Growth	Back color (JIS*)	Aerial hyphae and color	Soluble dye
Yeast extract / malt extract agar (ISP NO. 2)	Good	bright grayish yellow (5Y 7.0/3)	thick cotton-like, very pale yellow (5Y 9.0/3)	very pale yellowish red (8yr 5.5/11.5)
Oat meal agar (ISP NO. 3)	appropriate	bright grayish yellow (5Y 7.0/3)	slightly particulate, very pale yellow (5Y 9.0/3)	very pale yellow (5Y 9.0/3)
Starch/ Inorganic salt agar (ISP NO. 4)	Good	bright grayish yellow (5Y 7.0/3)	slightly particular, very pale yellowish red (8YR 8.0/2)	very pale yellowish red (8YR 5.5/1.5)

Tyrosine agar (ISP NO. 7)	appropriate	bright grayish yellow (5Y 7.0/3)	slightly particulate, very pale yellow (5Y 9.0/3)	very pale yellow (5Y 9.0/3)
Sucrose/ Nitrate agar (Waksman No.1)	appropriate	very pale yellow (5Y 9.0/3)	slightly particulate, white (N 9.5)	very pale yellow (5Y 9.0/3)
Glucose/ Asparagine agar (Waksman NO.2)	appropriate	very pale yellow (5Y 9.0/3)	slightly particulate white (N 9.5)	very pale yellow (5Y 9.0/3)
Nutrient agar (Waksman NO.14)	Good	very pale yellow (5Y 9.0/3)	thick, cotton-like white (N 9.5)	pale yellow (5Y 9.0/3)
Starch synthetic agar	Poor	very pale yellow (5Y 9.0/3)	slightly particulate white (N 9.5)	very pale yellow (8YR 8.5/1.5)
Potato/ Dextrose Agar	Good	very pale yellow (5Y 9.0/3)	abundant, cotton-like very pale yellow (2Y 9.0/3)	very pale yellow (5Y 9.0/3)

* The Standard Dye Table according to JIS Z8721 (Japanese Standards Association) was adopted.

2. Growth state on various culture media

The strain was cultured on the various culture media shown in Table 1, at a temperature of 27 °C for 7 to 14 days. Consequently, the strain OPC-21 grew very well on the yeast extract/malt extract agar culture medium (ISP NO. 2) and the starch/inorganic salt agar culture medium (ISP NO. 4). The growth was appropriate on the tyrosine agar culture medium (ISP NO. 7) and the glucose/sparagine culture medium. But the strain hardly grew on the starch synthetic agar culture medium. The growth state as shown in Table 1 was observed.

3. Physiological properties

a. Growth temperature and pH

Good growth at 27 to 32 °C, with the optimal pH of 8 to 10.3.

b. Starch degradation: positive

c. Milk coagulation: negative

d. Melanin dye generation: negative

e. Utilization and decomposition ability of carbon sources:

Various carbon sources were independently added to the Pridham• Gottlieb agar culture medium (ISP NO. 9) to 1 %. Then, the assimilability was observed.

D-glucose: great utilization

Inositol: no utilization

Lactose: utilization

D-mannitol: utilization

D-mannose: slight utilization but not so clearly

D-raffinose: no utilization

L-rhamnose: no utilization

Sucrose: no utilization

D-xylose: great utilization

4. Cell wall composition

By the method of Lechevalier et al. (The chemotaxonomy of actinomycete. In: Actinomycete taxonomy (Dieiz, A & D. M. Thater ed., Society for Industrial Microbiology, Virginia, pp. 225 (1980)), analysis of 2,6-diaminopimeric acid in the bacterial cell of the strain OPC-21 was done. It was shown that the compound was of meso type and cell wall type III/C type, with no characteristic reducing sugar or mycolic acid.

The phospholipid was of PIII type.

Based on the properties described above, the strain OPC-21 was determined as a bacterium (actinomycete) of the genus Nocardiopsis and was designated Nocardiopsis sp. strain OPC-21.

Advantages of the Invention

Actinomycetes generating a wide variety of secondary metabolites are an important bacterial group in practical sense. In accordance with the invention, the alkali protease

generated by the alkalophilic actinomycete can be purified. The alkali protease generated by the alkalophilic actinomycete when efficiently isolated can enlarge the applicable range thereof.

Furthermore, related-art enzymes with activities in ultimate states are mainly produced by thermophilic bacteria. However, the protease recovered in accordance with the invention is thermoresistant and has the optimal pH in alkalinity. From such respect, comparison with enzymes from general living organisms or examination using mutants thereof in terms of relation between substitution for amino acid residues and activity can bring about numerous findings about the enzyme structure and function or effective utilization of the enzyme.

Examples

The present invention will be described more specifically in the following examples.

Example 1

Isolation of OPC-210

An alkalophilic actinomycete was isolated from soil in Kyoto-fu in November 1987 according to the method of Horikoshi et al. (K. Horikoshi, Agric. Biol. Chem., 35, 1407 (1971)).

Culture conditions

In a culture medium of the following culture composition, pH 10.0 was cultured the strain under shaking at 120 rpm and a 7-cm shaking width at a culture temperature of 27 °C, for 2-day seed culture and 5-day subsidiary culture. The composition comprised 1 % by weight of glucose, 1 % by weight of casein, 0.5 % by weight of yeast extract, 0.1 % by weight of K_2HPO_4, 0.02 % by weight of $MgSO_4 \bullet 7\ H_2O$, and 1 % by weight of Na_2CO_3.

Purification of alkali protease and various properties

To a culture supernatant of the strain OPC-21 was added a 3-fold volume of cold

acetone, to recover an acetone powder. The powder (30.0 g) was dissolved in 20.0 ml of 10 mM CH_3COONH_4, pH 6.6. The resulting supernatant was dialyzed overnight against the same buffer. Subsequently, the dialyzate was subjected to chromatography on a DEAE-Sephadex A-50 column (Sephadex is a trade name of Pharmacia, Co.) equilibrated with the same buffer. The fraction passing through the column with no adsorption was defined Fr-1. For purification, Fr-1 was subjected twice to chromatography on CM-Sepharose CL-6B column (Sepharose is a trade name of Pharmacia Co.; the column was of a dimension of 1.9 × 50.0 cm) equilibrated with 10 mM CH_3COONH_4, pH 5.0 containing 0.1 M NaCl (Protease 1).

The individual purified fractions are shown in Table 2.

Fraction	Volume in ml	Protein[a)] in mg	Total activity[b)] in units	Specific activity units/mg
Culture supernatant	1800		431145.0	
Acetone powder	330	1518.0	183955.0	121.2
DEAE-Sephadex A-50	260	81.9	47634.9	581.6
CM-Sepharose CL-6B (first)	38	10.6	28510.5	2675.3
CM-Sepharose CL-68 (second)	33	1.5	18291.0	12194.0

a. Protein was determined by the Lawrey's method.

b. It was defined that one unit of the enzyme activity generated 1.0 μg of tyrosine per 1.0 ml • enzyme solution per one minute.

The protease 1 was recovered as a homogenous specimen at electrophoretic purity. Further, various properties of the enzyme were examined. The following results were obtained.

i. Molecular weight: about 21,000 (SDS electrophoresis, K. Weber, M. Osborn, J. Biol. Chem., 244, 4406 (1969))

ii. Optimal pH: 10 to 12 (casein as substrate)

iii. Optimal temperature: 60 to 70 °C (casein as substrate)

iv. Thermostability: the strain was stable up to 50 °C at pH 10 when treated for 30 minutes, but was completely inactivated at 70 °C.

v. pH stability: 80 % or more of the activity remained when treated at pH 4 to 8 and 60 °C for 30 minutes. However, the activity was nearly lost at pH 10.

vi. Stabilization: about 25 % of the activity remained when treated at pH 2 and 50 °C for 150 minutes. In the presence of 1 mM Ca^{2+}, however, the enzyme was stabilized at a level of 50 %.

vii. Effect of inhibitor: 2 mM PCMB and 10 mM EDTA never inhibited the activity.

Identification of bacterial strain

According to the ISP method (E. B. Shirling and D. Gottlieb, Int. J. Syst. Bacteriol., 16, 313 (1966)), the bacteriological properties (enzyme properties in culture and physiological-biochemical properties) were examined. The chemical taxonomy (diaminopimeric acid, reducing sugar, mycolic acid and phospholipid) was examined according to the method of Lechevalier et al. (M. P. Lechevalier and H. A. Lechevalier, Int. J. Syst. Bacteriol., 20, 435 (1970)). The results are shown in Tables 3 and 4.

Table 3

Chemical taxonomic properties	Characteristic properties
Cell wall type	III/C meso-A_2pm No characteristic reducing sugar
Cell wall lipid	PIII No mycolic acid

Table 4

Physiological properties	Characteristic properties
Action on milk	No coagulation, great peptidization
Melanin generation (tyrosine agar and yeast malt agar)	None
Starch hydrolysis	Positive
Growth temperature	Good growth at 27 to 32 °C
Growth pH	Good growth at pH 8 to 10.3

Herein, the protease 1 was completely inhibited by 5 mM PMSF. Thus, it was demonstrated that the protease was serine protease.